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## RESEARCH ARTICLE

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# Freshening increases the susceptibility to heat stress in intertidal mussels (*Mytilus edulis*) from the Arctic

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**Handling Editor:** Eoin O'Gorman**Abstract**

1. Temperatures in the Arctic are increasing at a faster pace than at lower latitudes resulting in range expansion of boreal species. In Greenland, the warming also drives accelerating melt of the Greenland Ice Sheet resulting in more meltwater entering Greenland fjords in summer.
2. Our aim was to determine if increasing summer temperatures combined with lower salinity can induce the expression of stress-related proteins, for example, heat shock protein, in boreal intertidal mussels in Greenland, and whether low salinity reduces the upper thermal limit at which mortality occurs.
3. We conducted a mortality experiment, using 12 different combinations of salinity and air temperature treatments during a simulated tidal regime, and quantified the change in mRNA levels of five stress-related genes (*hsp24*, *hsp70*, *hsp90*, *sod* and *p38*) in surviving mussels to discern the level of sublethal stress.
4. Heat-induced mortality occurred in mussels exposed to an air temperature of 30°C and mortality was higher in treatments with lowered salinity (5 and 15‰), which confirms that low habitat salinity decreases the upper thermal limit of *Mytilus edulis*. The gene expression analysis supported the mortality results, with the highest gene expression found at combinations of high temperature and low salinity.
5. Combined with seasonal measurements of intertidal temperatures in Greenland, we suggest heat stress occurs in low salinity intertidal area, and that further lowered salinity in coastal water due to increased run-off can make intertidal bivalves more susceptible to summer heat stress. This study thus provides an example of how different impacts of climate warming can work synergistically to stress marine organisms.

**KEYWORDS**

blue mussels, Greenland, heat shock proteins, littoral, multiple stressors, salinity, temperature, thermal stress

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## 1 | INTRODUCTION

The latest decade has been the warmest on record, and Arctic temperatures have risen at two times the global average (AMAP, 2019; Wassmann et al., 2011). Arctic warming accelerates the melting of glaciers, sea ice and the Greenland Ice Sheet, creating a feedback loop, as the ice and snow cover gradually loses its albedo effect. The Arctic coastline in general receives 11% of the world's riverine freshwater discharge, which combined with a decrease in Arctic sea ice extent results in an annual freshening of the Arctic ocean by 600 km<sup>3</sup> of freshwater (Fichot et al., 2013; Rabe et al., 2014). Since the 1980s, the mass loss of the Greenland Ice Sheet has increased sixfold, equivalent to an average net mass loss of 280 Gt per year (Morlighem et al., 2017; Mouginot et al., 2019). Thus, in Greenland fjords, the input of freshwater from melting ice has been observed to cause decreasing salinity and form distinct salinity gradients (Middelbo et al., 2019; Sejrs et al., 2017), where inner fjord surface water salinity can be as low as 5‰ (Meire et al., 2017; Sejrs et al., 2014).

Marine heatwaves are also expected to increase in both frequency, duration and intensity, with the largest changes projected to occur in the Arctic (Frölicher et al., 2018). Marine heatwaves are extreme events caused by periods of warm weather resulting in elevated sea surface temperatures (Frölicher et al., 2018). The detrimental effects of marine heatwaves have previously been observed in the Mediterranean, during a heatwave in 2003, where mass mortality among benthic marine organisms was observed (Garrahou et al., 2009). Intertidal species are, furthermore, shifting poleward in response to heatwaves (Sanford et al., 2019). For example, the southern distribution edge of *Mytilus edulis* in North America has shifted northwards by 350 km in the past 50 years due to a high mortality in southern populations as a direct effect of increased temperatures (Jones et al., 2010), and a recent marine heatwave in the northeast Pacific triggered a range expansions of 37 intertidal species towards the Arctic (Sanford et al., 2019). The effect of air temperature on body temperature during low tide is modified by the physical surroundings of the local habitat. Sessile intertidal organisms, such as blue mussels (*Mytilus* sp.), are subjected to varying temperatures, depending on their position in the intertidal zone, the substrate slope, orientation relative to the sun and shading by kelp (Helmuth et al., 2016; Kearney et al., 2014; Seed & Suchanek, 1992; Sejrs et al., 2021). In some habitats, individuals may be sheltered during low tide, in crevices or between boulders, where the effects of heat have less of an impact. The small-scale habitat variability of intertidal shores results in mosaic patterns of heat exposure resulting in local cold- and hotspots (Helmuth et al., 2006).

Blue mussels (*Mytilus* sp.) are freeze-tolerant conspicuous inhabitants in West Greenland fjords (Blicher et al., 2013; Thyrning et al., 2017; Thyrning, Juhl, et al., 2015). Blue mussels can survive subzero water temperatures (Thyrning, Rysgaard, et al., 2015), thus the northern distribution edge of intertidal blue mussels is restricted by extreme low aerial winter temperatures; blue mussels

are only found in intertidal microhabitats that reduce exposure to extreme temperatures (Thyrning et al., 2017, 2020). However, with increased warming, the number of days below the lower lethal temperature limit for *M. edulis* has decreased by ~57% (Thyrning et al., 2017). A local and poleward range expansion of blue mussels due to increasing temperatures is therefore a plausible future scenario in Greenland. Still, the positive effect of fewer extreme low temperature events during winter may be counteracted by an increase in extreme high temperature events during summer. The upper air temperature inducing significant mortality in North American *M. edulis* populations is approximately 32°C, a temperature at which a high rate of mortality in a population occurs when several consecutive exposures occur (Jones et al., 2010). In Greenland, summer temperatures surpassing 36°C in the intertidal zone have previously been reported (Høgslund et al., 2014; Thyrning et al., 2017), making scenarios in which high temperature induces heat stress or even mortality plausible. The consequence of high temperature events for *M. edulis* in Greenland may be exacerbated by the lowered salinity of coastal waters due to increased ice melt. Decreased salinities change the shell production and mineral composition of *M. edulis* (Telesca et al., 2018, 2019), and reduce the growth rate as the costs of calcification are increased (Sanders et al., 2018). The growth of soft tissues is also reduced due to allocation of energy to osmoregulation and cell repair (Landes et al., 2015). An effect of combined temperature and salinity stress has already been established on other organisms. Diehl et al. (2020) showed that high temperature and low salinity in Arctic fjords have a negative impact on the physical and biochemical status of the Arctic brown seaweed *Laminaria solidungula*, and low salinity-high temperature stress has proven to increase mortality in Baltic blue mussels (Hiebenthal et al., 2012).

One physiological response to environmental stress is an increased production of stress-related proteins such as heat shock proteins. It is generally acknowledged that an upregulation of stress-responsive proteins occurs in *M. edulis* as a response to the cellular damage inflicted by high temperatures (Buckley et al., 2001; Jones et al., 2010; Lockwood et al., 2010; Paul Chapple et al., 1997). However, experiments have shown that the induction of stress-responsive proteins not only occurs when mussels are subjected to high temperatures, but also to low salinities (Hamer et al., 2004; Podlipaeva & Berger, 2012). This suggests that exposure to both low levels of salinity and high temperature simultaneously might have synergistic effects on the stress level applied to the organism, the expression of the heat shock response and mortality.

In this study, we test if summer temperatures in Greenland can induce mortality and/or expression of heat shock proteins in intertidal mussel and to what extent salinity influences the cellular stress response to high temperatures. To answer this question, we experimentally determined the air temperature that induces mortality and measured the stress protein expression level prior to mortality occurring in a full factorial combination of lowered salinity and high temperature exposures. To mimic natural conditions, animals were

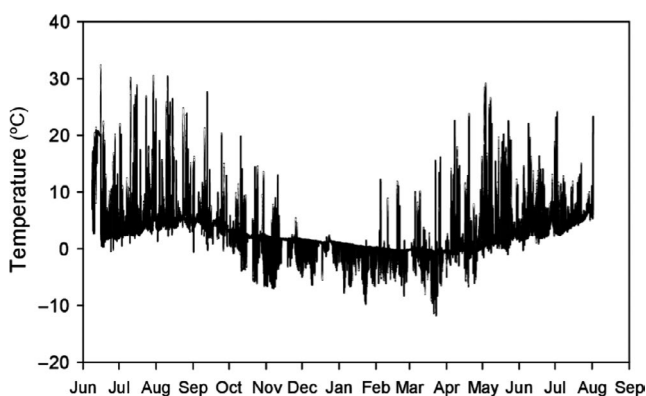
exposed to repeated simulated tidal cycles, with high tide exposing subjects to controlled salinities and low tide exposing subjects to controlled air temperatures. Combined with in situ intertidal temperature measurements from data loggers, we discuss the potential influence of heat stress in *M. edulis* as an Arctic intertidal keystone species.

## 2 | MATERIALS AND METHODS

### 2.1 | Sampling

Blue mussels (*M. edulis*) were collected from a pure *M. edulis* population (Mathiesen et al., 2017) in the lower intertidal zone of a gravel beach in Kobbefjord south of Nuuk, Greenland (64°10'50.8"N, 51°32'28.1"W) on the 5 August 2019. A total of 700 mussels with a shell size range of 30–50 mm were collected, along with ~100 L of seawater. The mussels were kept in a cold storage room (5°C) and divided into four 10-L aquaria with a constant supply of air and a daily change of seawater for 3 days. The mussels were kept in a cooler box with wet towels and transported to holding facilities at Aarhus University. Upon arrival, mussels were divided into four aquariums with a constant supply of air and a daily change of seawater with a salinity of approximately 23‰, corresponding to the salinity at the collection site.

To estimate microhabitat-level air temperatures in the intertidal zone, we deployed five TidbiT Robomussel temperature loggers in different parts of Godthaabsfjord, Nuuk, approximately 1 year prior to our sampling (Helmuth et al., 2016). Due to ice scouring, only one functional logger was retrieved near Kapisillit in the inner part of the fjord (64°27'03"N, 50°19'03"W). The logger, which recorded air and water temperature, was placed in a mussel bed in the intertidal zone. Data were logged from the 8 June 2018 until the 1 August 2019, with a resolution of 30 min (Figure 1). The maximum intertidal air temperature recorded was 32.4°C while the lowest recorded air temperature was -11.7°C. The water temperature varied from 0 to 7°C.



**FIGURE 1** Microhabitat temperatures measured every 30th minute within the intertidal zone in Kapisillit, Godthaabsfjord, Greenland from June 2018 to August 2019

### 2.2 | Experimental design

We exposed mussels to experimental air temperatures of 5, 30, 33 and 36°C combined with salinities of 5‰, 15‰, 23‰, with 5°C and 23‰ representing control conditions. The highest experimental temperature was 0.6°C above the maximum observed temperature from the in situ logger (Figure 1), demonstrating the relevance of the 33°C laboratory experiment. Furthermore, short-term intertidal maximum temperatures of 36°C have been recorded elsewhere in Greenland (Thyrring et al., 2017). Surface salinity in inner fjords is often below 15‰ in summer (Sejr et al., 2014, 2021) and since large aggregations of mussels can be found in river mouths (Duarte et al., 2020), we consider the exposure to salinities of 5‰ to represent realistic albeit extreme conditions. Combined, the experimental conditions were designed to represent extreme current conditions, that are expected to influence a larger proportion of the mussel populations in Greenland and for increased duration in the future.

All collected mussels were subjected to a 1-week adjustment period after arrival in Aarhus, during which the mussels were exposed to air two times a day at 12-hr intervals for 1.5 hr each time, to simulate a semi-diurnal tide. The water and air temperature for the adjustment period was 5°C. The adjustment period was used to ensure that mussels had adjusted to the laboratory conditions prior to the actual experiment starting. No mortality among mussels occurred during the adjustment period. The mussels were fed every second day with 5 ml of algae solution (Shellfish diet 1800, Reed Mariculture). A total of 480 individuals with a shell length of 30–40 mm were selected and distributed into forty-eight 0.5-L buckets in which holes were drilled to allow water to pass through. The buckets each contained 10 mussels subjected to similar experimental treatments. Buckets were distributed to 12 aquaria (10 L), with four aquaria for each salinity regime (5‰, 15‰ and 23‰ [control]). The desired salinities were reached by diluting the 23‰ seawater with demineralized water and measuring the salinity with a digital refractometer.

The experiment began on the 17th August at 18.30 and ended 6 days later on the 23rd August at 06.30. A simulated semi-diurnal tide was created every 12th hour for 1.5 hr during the course of the experiment, by taking the buckets out of the water and placing them in thermal-controlled incubators at 5, 30, 33 and 36°C respectively. During the 6-day experimental period, specimens were subjected to a total of 12 cycles of 1.5-hr exposure to air temperatures. Subjecting the mussels to these air temperatures and the three salinities resulted in a total of 12 treatments. Each individual bucket containing 10 mussels was subjected to the same treatment for the entire course of the experiment, while each treatment had four replicate buckets, totaling 40 mussels per treatment. To document that each treatment was subjected to the desired temperatures over the course of the experiment, iButton temperature loggers (iButtonlink Technology) were placed in a random bucket from each temperature regime, where it measured the temperature every 10th minute for the duration of the experiment (Supporting Information Figures S1–S4). Prior to each temperature treatment, dead mussels were collected, the shell length was

measured with a digital caliper and the time of death was noted. Once a day, during the temperature treatment, the water in all 12 aquaria was changed. A small amount (5 ml) of algae solution (Shellfish Diet 1800, Reed Mariculture) was added to each aquarium every second day to avoid starvation during the experiment.

After the last simulated low tide exposure, mussels were left overnight in aquaria after which the shell length of surviving mussels was measured, and gill tissue was dissected and instantly frozen using dry ice. Gill tissue samples were stored at  $-80^{\circ}\text{C}$  and used for the following gene expression analysis. Mussels subjected to the  $36^{\circ}\text{C}$  treatment were excluded in the gene expression analysis, as the mortality in this treatment was  $\sim 100\%$ .

## 2.3 | Gene expression analysis

We investigated gill tissue of *M. edulis* from nine treatments (full factorial design of the two factors: salinity (5‰, 15‰, 23‰) and temperature (5, 30,  $33^{\circ}\text{C}$ ). We used six to nine individuals from each treatment ( $N_{\text{tot}} = 65$ ). RNA extraction was performed using the RNeasy Protect Mini kit (Qiagen) according to the manufacturer's instructions. The resulting amount of total RNA was quantified (Invitrogen Qubit<sup>TM</sup> fluorometer) and cDNA was synthesized from 1,000 ng total RNA from each sample (Affinity Script cDNA Synthesis Kit, Agilent Technologies) according to the manufacturer's instructions, diluted to a concentration of 4 ng/ $\mu\text{l}$  and stored at  $-20^{\circ}\text{C}$ . Gene expression of mussel gill tissue samples was quantified using real-time qPCR on a Stratagene MxPro - MX3005P qPCR system (AH Diagnostics) as described in Waagner et al. (2013). The genes tested were as follows; *hsp70*, *hsp24*, *hsp90*, *p38* and *sod*

(Table 1). Amplification plot and dissociation curves were inspected to identify occasional low-quality PCR runs (which were then discarded) and data were converted to linear  $R_0$  values using DART-PCR v1.0 (Peirson, 2003). Gene expression data were normalized with NORMA-Gene (Heckmann et al., 2011).

## 2.4 | Statistics

All statistical analyses were conducted using R (R Core Team, 2020). A GLM with a binomial distribution and a logit link function was used to test for significant difference in mortality proportion with temperature and salinity as the dependent variables. A test for overdispersion was done by dividing the residual deviance with residual degrees of freedom.

A two-way ANOVA was used to test for significant differences in gene expression with temperature and the salinity as the dependent variables. Visual inspection of Q-Q plots and the residual distribution revealed that log transformation of the gene expression data adequately improved the distribution of the data to justify the ANOVA analyses. The level of significance in both mortality and gene expression was  $p\text{-value} < 0.05$ .

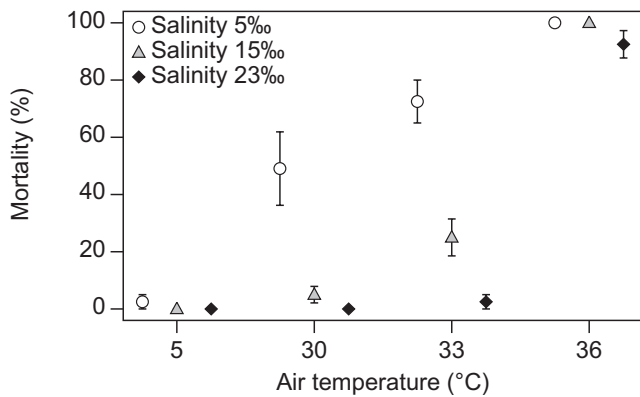
## 3 | RESULTS

### 3.1 | Mortality experiment

We found a significant effect of salinity ( $F_{1,44} = 21.26$ ,  $p < 0.001$ ), temperature ( $F_{1,44} = 86.44$ ,  $p < 0.001$ ) and their interactions

**TABLE 1** Genes tested in the gene expression analysis, their expected target response and primer sequences. The length of primers is 20–21 bp

Gene	Expected target response	Primer sequence	Reference
<i>hsp90</i> : Heat shock protein 90 kDa	Temperature	Forward primer: TCAGCAACAAGGTAAGCGGA Reverse primer: TCATGGAGCTCTTCAAGCTG	(GenBank: JZ970419.1) (Buckley et al., 2001)
<i>sod</i> : Superoxide dismutase	Oxidative stress	Forward primer: AGCTATCCCTGACTGGTCCC Reverse primer: CATGGCCACCACCTTTACCT	(GenBank: AJ581746.1) (Mlouka et al., 2019; Regoli et al., 1997)
<i>hsp70</i> : Heat shock protein 70 kDa	Temperature/salinity	Forward primer: AACTGCTGAAGCGTATCTGGG Reverse primer: CCTGCAATGAAACCAGCATCC	(Granger Joly de Boissel et al., 2017; Lockwood et al., 2010; Podlipaeva & Berger, 2012)
<i>hsp24</i> : Heat shock protein 24 kDa	Temperature/salinity	Forward primer: AGATGACAGTTCCACGGTCTG Reverse primer: TGCCCGGATAGTAAGATTGCC	(Granger Joly de Boissel et al., 2017; Lockwood et al., 2010; Lockwood & Somero, 2011; Fields et al., 2012)
<i>p38</i> : Mitogen-activated protein kinases	Salinity	Forward primer: CATTGGAGCGAATCCTCTTGC Reverse primer: GCATCTTCTGCAGTGACACG	(Granger Joly de Boissel et al., 2017; Hamer, 2008)



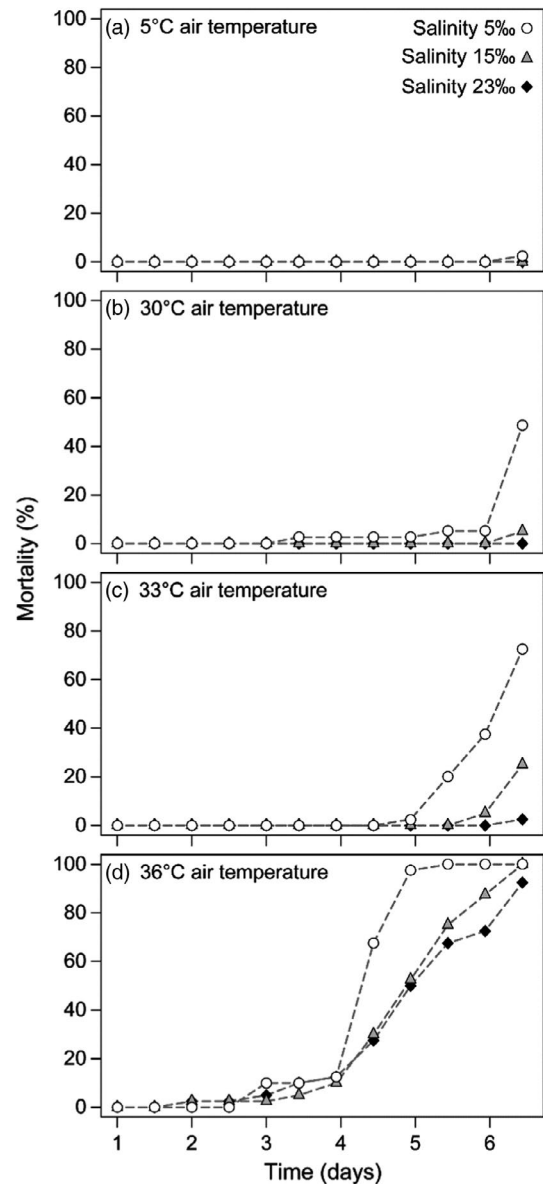
**FIGURE 2** Mean mortality (%) in blue mussels (*Mytilus edulis*) subjected to 12 cycles of 1.5-hr simulated low tide. Mussels were exposed to four different air temperatures (5, 30, 33 and 36°C) during simulated low tide. While submerged, the mussels were exposed to three different salinities (5‰ (○), 15‰ (Δ), 23‰ (◆)) with a water temperature of 5°C. The experiment ran for six consecutive days. Error bars signify standard error

( $F_{1,44} = 14.97$ ,  $p < 0.001$ ) on survival. Air exposure at high temperatures (33–36°C) and control salinity (23‰) produced a mean mortality of 2.5% and 92.5%, respectively, while no mortality occurred in the 5 and 30°C temperature treatments (Figure 2; Table S1). Thus, the upper thermal limit for Greenland *M. edulis* is approximately 36°C under common saline conditions. Low salinity (5‰) at the control temperature (5°C) resulted in 2.5% mortality (Figure 2); however, when subjected to temperatures  $\geq 30^\circ\text{C}$  and salinities  $< 23^\circ\text{C}$  simultaneously, a negative correlation occurred. Most pronounced was the results from the lowest salinity treatment (5‰), showing an increase in mortality with increasing air temperatures (30, 33, 36°C) from 49% and 72.5% to 100%, suggesting a synergetic interaction between high air temperatures and low salinity conditions (Figure 2), which clearly depressed the upper thermal limit.

When mortality over time was examined (Figure 3), it became evident that exposure time also had an effect. The results showed that following four days of immersion and emersion cycles with air temperatures exceeding 33°C, a rapid increase in mussel mortality occurred (Figure 3c,d). At 36°C, the air temperature in itself was enough to induce mortality after only six 1.5-hr exposures (Figure 3d). The mortality at low salinities, especially 5‰ and 15‰ increased at a higher rate than at 23‰ (Figure 3c,d). These results suggest that, had the experiment been allowed to continue, a similar trend in temperature–salinity produced mortality could occur in treatments with lower levels of temperature- and salinity stress.

### 3.2 | Gene expression analysis

Expression of heat shock proteins and other stress-responsive genes was clearly regulated by high temperatures, while the upper temperature at which production occurred was modified by salinity.



**FIGURE 3** Mortality (%) in blue mussels (*Mytilus edulis*) as a function of time (days). Mussel were exposed to four different temperatures during emersion (a: 5°C, b: 30°C, c: 33°C and d: 36°C) and three salinities during submersion (5‰ (○), 15‰ (Δ) and 23‰ (◆)) in 5°C water. Exposure to experimental air temperatures during emersion was done 12 times each for a period of 1.5 hr. The experiment ran for six consecutive days. Mortality was monitored before each emersion treatment

There was a significant difference in the expression of *hsp90*, *hsp70* and *hsp24* among temperature treatments (Table 2; Figure 4a–c), and low salinity furthermore significantly affected the gene expression of *hsp70* (Figure 4b). No significant interactions were found among the two variables for any of the investigated *hsps* (Table 2). The fold change in gene expression increases with increasing temperatures and decreasing salinities; however, there was a tendency towards a higher fold change at 15‰ at both 30 and 33°C (Figure 4). No significant differences were found in the expression of *sod* or *p38* (Table 2); however, our results show a clear tendency towards an

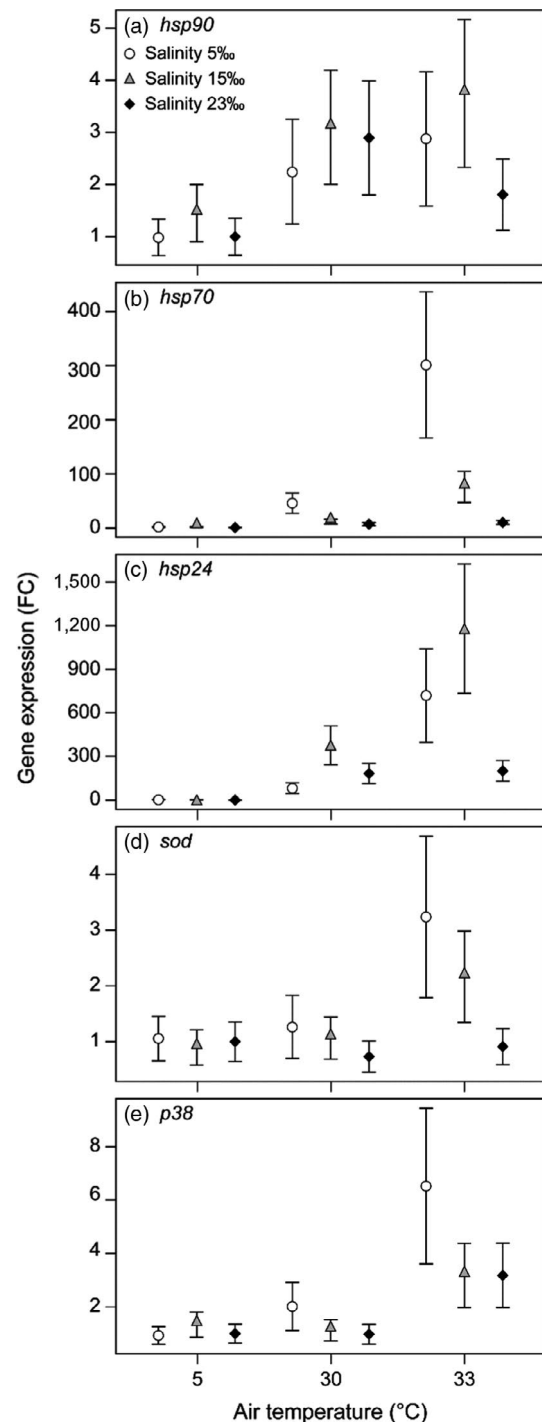
**TABLE 2** Results of the two-way ANOVA for the expression of genes (*hsp70*, *hsp24*, *hsp90*, *p38* and *sod*) in blue mussels (*M. edulis*), subjected to 12 cycles of 1.5-hr emersion. Mussels were exposed to four different air temperatures (5, 30, 33 and 36°C) during a simulated low tide for six consecutive days. While submerged, the mussels were exposed to three different salinities (5‰, 15‰, 23‰) with a water temperature of 5°C. Data have been normalized and log transformed

	<i>df</i>	Mean sq	<i>F</i> value	Pr(> <i>F</i> )
<i>hsp90</i>				
Salinity	2	0.61	1.16	0.32
Temperature	2	6.28	11.95	<0.001
Salinity: temperature	4	0.41	0.77	0.54
Residuals	52	0.51		
<i>sod</i>				
Salinity	2	1.78	1.66	0.19
Temperature	2	1.94	1.82	0.17
Salinity: temperature	4	1.58	1.48	0.22
Residuals	54	1.06		
<i>hsp70</i>				
Salinity	2	16.45	11.66	<0.001
Temperature	2	66.55	47.16	<0.001
Salinity: temperature	4	1.66	1.18	0.33
Residuals	52	1.41		
<i>hsp24</i>				
Salinity	2	6.79	2.00	0.15
Temperature	2	78.45	23.12	<0.001
Salinity: temperature	4	3.703	1.09	0.37
Residuals	55	3.39		
<i>p38</i>				
Salinity	2	1.59	1.12	0.33
Temperature	2	2.90	2.05	0.14
Salinity: temperature	4	2.63	1.86	0.13
Residuals	53	1.41		

increased expression of both *sod* and *p38* when high temperatures and low salinities co-occur (Figure 4d,e).

## 4 | DISCUSSION

The rocky intertidal zone is an important model system for marine ecologists and provides examples of the long-term impacts of a warming climate (Paine, 1966; Sorte et al., 2017). In the intertidal zone, mussels can be the dominant species and thus impact community and food web structure (Paine, 1974; Sorte et al., 2017). Therefore, identifying factors that directly impact mussels' physiological responses to thermal stress increases our capacity to predict ecosystem-wide impacts of continued warming. In the Arctic, increasing temperatures are expected to benefit boreal species and allow them to expand northwards. For mussels, an Arctic range



**FIGURE 4** Change in gene expression (fold change, FC) in blue mussels (*Mytilus edulis*) for genes *hsp90* (a), *sod* (b), *hsp70* (c), *hsp24* (d) and *p38* (e) in nine different treatments, consisting of three air temperatures during emersion (5, 30 and 33°C) and three salinities (5‰ (○), 15‰ (△) and 23‰ (◆)) at 5°C during submersion. Fold change values are given relative to control treatment (5°C, 23‰). Error bars signify standard errors

expansion has been observed (Berge et al., 2005) and their current northern intertidal limits have been attributed to mortality from freezing during winter low tides. However, it is predicted that blue mussels will increase in abundance and expand upwards in the



intertidal zone as the number of days with temperatures below their lower thermal limit is decreasing (Thyrring et al., 2017). In this study, we wanted to test if the low salinity typically found in Greenland fjords during summer would make bivalves more susceptible to heat-induced mortality and induce increased expression of stress-related proteins to assess if increasing summer temperatures in Greenland could limit the distribution of mussels in the intertidal zone. Three main findings emerged: (a) low salinity increased mortality in mussels when exposed to high temperatures, (b) increased expression of heat shock proteins at air temperatures  $\geq 30^{\circ}\text{C}$  while one gene (*hsp70*) also responded to low salinity and (c) intertidal temperature data from Greenland suggest that lethal and sublethal heat stress can occur and is likely to be more frequent in the future.

#### 4.1 | Upper thermal limit and salinity

Intertidal *M. edulis* is highly resilient to thermal stress (Clark et al., 2021), but the current study shows that the upper thermal limit of *M. edulis* is modified by habitat salinity. Although the upper thermal limit of *M. edulis* in Greenland is approximately  $36^{\circ}\text{C}$  under control salinity conditions (when exposed for 1.5 hr diurnally), the upper thermal limit was significantly decreased when exposed to lower salinity conditions (Figure 2). Low salinity reduced both the air temperature at which mortality occurred and the exposure time required to induce mortality (Figure 3). Consecutive aerial exposures have been proven to reduce the thermal tolerance of blue mussels, indicating that repeated exposures to high temperatures induces a mortality response in blue mussels (Jones et al., 2009; Seuront et al., 2019; Sorte et al., 2019). This coincides well with our mortality results, in that mortality rapidly increases after multiple exposures to high temperature (Figure 3c,d).

An interactive effect of low salinity and high temperature has previously been reported in other organisms (Southworth et al., 2017). In *M. edulis*, the combined effect of both stressors decrease the survival of larvae, the shell formation and the general condition (Brenko & Calabrese, 1969; Hiebenthal et al., 2012). The present study is the first to demonstrate an interactive effect of low salinity and high aerial temperature on the mortality of *M. edulis*.

#### 4.2 | Expression of stress-responsive genes

The gene expression analysis demonstrates that low salinity affects the expression of heat shock proteins during high temperature stress. A significant increase in *hsp24*, *hsp70* and *hsp90* expression in response to high temperature was observed, while a significant increase in *hsp70* was recorded at low salinity (Table 2). The significant upregulation of *hsp70* production in response to both low salinity and high temperature proves it as a useful biomarker for measuring the combined effects of salinity and heat stress. In contrast, *hsp24* and *hsp90* seem to be less responsive to salinity effects

and are therefore probably best suited to measure thermal stress alone.

The upregulation of heat shock proteins as a response to high temperature in *M. edulis* coincides well with previous studies, which also showed an increase in heat shock protein production in *Mytilus* mussels as a response to thermal stress (Buckley et al., 2001; Jones et al., 2010; Lockwood et al., 2010; Paul Chapple et al., 1997). The increase in *hsp70* expression in response to low salinity supports previous results by both Hamer et al. (2004) and Podlipaeva and Berger (2012). Both found an increase in *hsp70* when blue mussels were subjected to low levels of salinity, confirming that salinity stress inflicts cellular stress. Our results show that high temperature ( $33^{\circ}\text{C}$ ) and low salinity clearly increases the production of *hsp70*, suggesting an interaction between the two stressors, though this was not supported by the statistical analysis. However, this is an interesting observation, as other intertidal studies have found very little overlap in gene expressed from heat and salinity stress (Lockwood et al., 2010; Lockwood & Somero, 2011).

The non-significant increase in the expression of *sod* and *p38* suggests an induction occurring as a response to unfavourable osmotic conditions (Hamer, 2008). The upregulation of stress-responsive genes at both salinity and temperature stress implies that the heat shock response is a mechanism used at a variety of unfavourable conditions, while in contrast, the minor induction of *sod* and *p38* suggests that these mechanisms are more specific and are not upregulated when heat and salinity stress co-occur. The effect of low salinity on the expression of stress-responsive proteins (here particularly heat shock proteins) adds to the knowledge that these stress-responsive proteins not only responds to thermal stress but may be modified by other factors (Sørensen, 2010), which complicates the interpretation of expression in field collected organisms.

We do note that our data on gene expression represent a single time point, at the end of the 6-day experiment. Induction of stress-responsive genes can occur rapidly, particularly at the transcriptomic level investigated here. Similarly, associated downregulation upon return to benign conditions also happens rapidly (Buckley, 2006; Sørensen et al., 2005). It has also been demonstrated how dynamic, in time and space, the expression of heat shock proteins can be under field conditions, especially in the intertidal (Gracey et al., 2008). Thus, it is possible (and even likely) that mussels in our study have shown similar dynamics with some or all of the investigated genes showing transient upregulation in response to the stress of salinity or temperature changes. Furthermore, the signal detected in our study represents the response to a more chronic stress condition. Regardless, the detected upregulation of stress-responsive genes clearly indicates a proportional stress exposure.

#### 4.3 | Ecological impacts of heat stress in Greenland

Our experiment shows that several days of exposure to temperatures of at least  $33^{\circ}\text{C}$  is required to induce substantial mortality

in intertidal mussels. This does not appear to be a common occurrence in Greenland, and intertidal mussel will thus mostly experience sublethal effects of heat stress. However, it appears conditions in Greenland are approaching a threshold for inducing mortality, which suggest that continued warming will increase the likelihood of mussel mortality induced by Arctic heatwaves or alternatively, that other regions in the Arctic already have crossed this threshold.

The upregulation of heat shock proteins in *M. edulis* by low salinity during submersion may well happen in natural populations in Greenland. Loggers placed in the intertidal zone measured temperatures well above 30°C (Figure 1), coinciding with recordings from South Greenland (Høgslund et al., 2014), and intertidal temperatures exceeding 36°C have been measured further north on Disko Island (Thyrring et al., 2017). With the continued increase in meltwater run-off to Greenland fjords, a combination of high temperature events and low salinity can co-occur in intertidal mussel habitats, potentially skewing the distribution of *M. edulis* to protective microclimate where temperatures remain below the upper thermal limit.

Blue mussels are one of the most abundant macrozoobenthic species in Greenland's intertidal (Thyrring et al., 2021), and the Godthaabsfjord supports populations of *M. edulis* in a wide range of salinities, and dense beds have been found in brackish lagoons and near river mouths where salinities are especially low (Duarte et al., 2020). A possible future scenario for the distribution of *M. edulis* in Greenland is a change in abundance from the innermost parts of fjords to the outermost, as temperatures further inland are higher than coastal temperatures, and the inner parts of fjords are the first to receive meltwater, thus having the lowest salinities (Meire et al., 2017). Such a distribution is already evident in Northwest Greenland, where *M. edulis* inhabits outer fjord habitats while *M. trossulus*, a congener known to be more tolerant of low salinities, inhabits inner fjord habitats (Wenne et al., 2016). In terms of abundance and distribution on a larger scale, higher temperatures may favour a northward shift as described by Thyrring et al. (2017), but a positive change in abundance due to fewer days below the lower thermal limit may be counteracted by the increase in days with temperatures surpassing the upper thermal limit (Thyrring et al., 2017). Thus, our study provides an important example of how two consequences of climate change combine synergistically to influence the thermal biology of a boreal species in the Arctic.

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## AUTHORS' CONTRIBUTIONS

This study is the product of the Master thesis of M.B.N. and T.K.V. who contributed equally and were supervised by M.K.S., J.G.S. and J.T.; The study was designed by M.K.S., J.G.S. and J.T.; M.B.N. and T.K.V. conducted the experiment, analysed the data, prepared the figures and wrote the first draft of the manuscript, which was subsequently improved with input from M.K.S., J.G.S. and J.T.

## DATA AVAILABILITY STATEMENT

Data for this study are freely available through the zenodo repository and can be accessed at <https://doi.org/10.5281/zenodo.4454510> (Nielsen et al., 2021).

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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